Conus venoms affect chemical signalling in the brain.

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RÉSUMÉ.

Parmi les gastéropodes marins, les cônes forment une large famille comprenant plus de 300 membres. Ils se nourissent de divers organismes tels qu' autres gastéropodes, vers ou poissons. Ils injectent un puissant venin dans leur victime, provoquant ainsi la paralysie quasi instantanée de ce dernier, ce qui permet au cône d'envelopper de son estomac extensible sa proie immobilisée. Bien que les cônes attaquent essentiellement dans le but de se nourrir, ils peuvent également agir de la sorte pour se défendre d'une éventuelle agression humaine ou animale. Le *Conus geographus* ainsi que le *Conus textile* sont même tenus pour responsables d'un certain nombre de décès humains, ce pourquoi, il est généralement conseillé de considérer tout cône vivant comme étant potentiellement dangereux.

Bien que récent, l'intérêt consacré aux mécanismes d'action et à l'utilisation thérapeutique des venins de cônes s'accroît rapidement. Dans les années soixante, Endean et son équipe furent les premiers à isoler les venins de cônes et à en étudier les effets sur différentes espèces animales (ENDEAN and RUDKIN, 1965). Ultérieurement, Kobayashi et ses collaborateurs (KOBAYASHI *et al.*, 1982) ont mené ce type de recherche sur des tissus et organes isolés et ont observés que ces venins agissaient différemment d' une espèce à l' autre. A la même époque, Olivera et son équipe isolaient différents peptides du venin de *Conus geographus* (OLIVERA *et al.*, 1985, 1990). Certaines de ces toxines (les α-, μ- et ω-conotoxines) affectent des processus cellulaires vitaux tandis que bien d' autres ne produisent aucun effet létal chez la souris. En ce qui concerne la composition de ces venins, nous pouvons constater une diversité interspécifique remarquable, celle-ci venant s'ajouter à la multiplicité des peptides présents dans un même venin.

Nous avons initialement émis l' hypothèse que les venins de cônes peuvent contenir des toxines qui agissent sur des récepteurs hormonaux et neurotransmetteurs. Nous avons en effet trouvé que le venin de cinq cônes (*C. planorbis*, *C. tessulatus*, *C. eburneus*, *C. textile* and *C. geographus*) contiennent des toxines qui sont capables d'agir (de manière "calcium-dépendante") avec les récepteurs α_2-adrénergiques dans la rétine de veau (CZERWIEC *et al.*, 1989), confirmant ainsi notre hypothèse de départ. Dans le cerveau de veau par contre, le venin du *C. tessulatus* reconnaît les récepteurs "5-HT_{1A}" sérotonergiques tandis que les récepteurs α_2-adrénergiques ne le sont pas (Fig. 3). Par conséquent, les récepteurs α_2-adrénergiques de la rétine semblent être différents de ceux présents dans le cerveau. Ceci indiquerait que les toxines de cônes peuvent différencier de façon sélective certains récepteurs étroitement liés. L'utilisation de toxines de cônes s'avère être un outil précieux afin de mieux comprendre divers processus cellulaires.

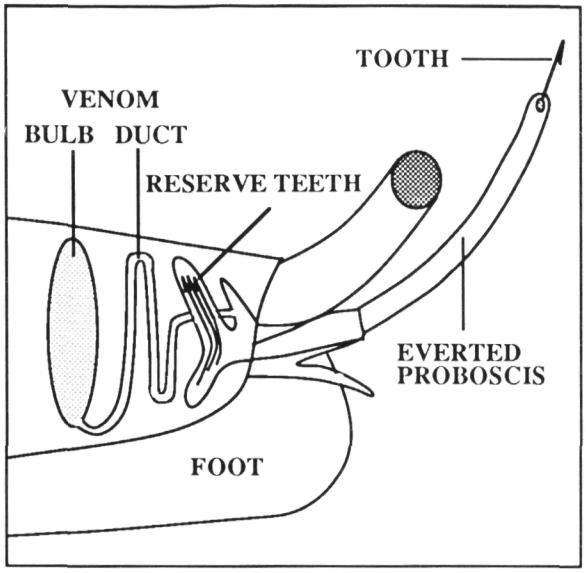

Fig. 1. Schematic representation of the venom apparatus of cone shells.

Introduction

Plant and animal toxins have proven to be extremely useful in defining key components of vital physiological systems. In this context, *Conus* venoms constitute a novel source of peptide toxins which are capable of interfering with the transmission of nerve signals by interacting with ion channels as well as with neurotransmittor receptors.

Conus venoms for feeding and defense.

The family Conidae consists of more than 300 members of marine snails. They possess potent venoms which are used primarily in the capture of prey organisms such as worms, other molluscs and fish (ENDEAN and RUDKIN, 1965). Regardless of the feeding type, all *Conus* species possess a similar, sophisticated venom apparatus (OLIVERA *et al.*, 1988). This apparatus is composed of a muscular venom bulb (which functions as a pump), a long hollow duct (where the venom is made), the pharynx with associated specialised sacs containing disposable, harpoon-like teeth (serving as hypodermic needles for the venom) and the proboscis (a tube forming part of the mouth) (Fig. 1). Like most predators of active animals, cones have a very sensitive sensory system enabling them of detecting the presence of a prey at some distance by "smelling" the water current passing through their gill chamber (MARSH and SLACK-SMITH, 1986). Feeding behaviour in the wild is only poorly documented, but observations from aquaria reveal that when a cone is ready to attack its prey, it transfers one of the teeth into the proboscis. The prey is then speared with the harpoon-like tooth held by the tip of the suddenly-everted proboscis (at least for *C. magus* Linnaeus, 1758 and *C. purpurascens* Sowerby, 1833) (OLIVERA *et al.*, 1988). At the same time, the muscular bulb is believed to contract, so that the venom is pushed trough the duct, the pharynx, the proboscis and finally through the hollow tooth into the victim. This provokes instant paralysis, so that the snail can engulf the immobile prey with its distensible stomach.

Although such attacks are made principally for feeding purposes, cones are also known to use their venom apparatus for defensive purposes. In this respect, piscivorous species such as *C. geographus* Linnaeus, 1758 and also molluscivorus species such as *C. textile* Linnaeus, 1758 pose a serious threat to vertebrates, including man (ENDEAN and RUDKIN, 1965; RICE and HALSTEAD, 1967) Both species have indeed been held responsible for several human deaths. In addition, many severe injuries have been caused by the stings of other *Conus* species and even vermivorous species possess venoms which produce local effects in vertebrates such as tissue necrosis and haemmorhage (ENDEAN and RUDKIN, 1965). It is therefore widely recommended to regard all living cones as dangerous and not to handle them with bare hands. Even holding the shell far away from the anterior end (which is considered to be the safest way) is not without potential risk since the extensible proboscis

may bend and reach as far back as the spire (MARSH and SLACK-SMITH, 1986).

Conus venoms: diversity and action mechanisms.

Interest in the action mechanism and potential therapeutic use of *Conus* toxins is only recent, but is rapidly growing. Endean and co-workers were, in the early sixties, the first to isolate crude *Conus* venoms from ducts and to investigate their effect on different animal species (ENDEAN and RUDKIN, 1965). Some interesting correlations could be found between the feeding habit of the cone and the lethal doses of their venom on certain animal groups. The venom of piscivorous cones was lethal for fish and mice but not for gastropods and polychaetes. On the other hand, the venom of vermivorous *Conus* species were only lethal for polychaetes.

The action mechanisms of these venoms became gradually unraveled since the early eighties. Kobayashi and coworkers (KOBAYASHI *et al.*, 1982) performed a comparative study of the venoms of 29 *Conus* species, by testing their effect on isolated tissues and organs (diaphragm, atria, ileum and aorta) of different mammals. Their results clearly illustrated that the effect of the venom on these tissues and organs could differ from one *Conus* species to another. This indicated that the chemical composition of the venoms might be dependent on the *Conus* species. In this context, it was found that the venom of *C. geographus* possess a curare-like toxin, that the vonoms of *C. magus* and *C. striatus* Linnaeus, 1758 contain a component that increases the sodium permeability of cardiac and nerve cell membranes, and that the venoms of *C. eburneus* Hwass, 1792 and *C. tessulatus* Born, 1778 possess a toxin that increases the calcium permeability of cardiac and smooth muscle membranes.

At about the same time, Olivera and coworkers were able to isolate different peptide toxins from the venom of *C. geographus* and *C. magus* (OLIVERA *et al.*, 1985). These studies revealed that the venom of each Conus species contains a range of toxins with different specificity of action, and that the nature and occurrence of these toxins is species-dependent. In this, and subsequent studies (OLIVERA *et al.*, 1988, 1990) much attention was given to three distinct types of conotoxins: i.e. the α-, μ-, and ω-conotoxins. These toxins interfere with cellular processes that nerve cells require to command the activity of skeletal muscles as well as with biochemical processes in the muscle cells themselves.

The correct functioning of certain muscles such as the heart and the diaphragm (which partly controls respiration) is absolutely necessary for survival of higher organisms. Contraction of such muscles is under the control of nerve impulses. Such impulses travel under the form of electric signals from the nerve cell body to the nerve endings, which make contact with target cells such as other nerves or muscle cells (ALBERTS *et al.*, 1989). This electric signal cannot be transferred to the target cells directly. Instead, when the electric signal arrives at the nerve ending, it will open voltage-sensitive calcium channels in the membrane. The resulting influx of calcium ions into the nerve ending then triggers the release of small molecules (i.e. neurotransmitters) into the small space (the synaptic cleft) between the nerve ending and the target cell (Fig. 2). The neurotransmitters will then bind to specific recognition sites (i.e. receptors) which are located at the surface of the target cells, and this interaction will induce a physiological response of these cells (Fig. 2). For example, the neurotransmitter acetylcholine triggers the contraction of skeletal muscles by interacting with nicotinic acetylcholine receptors at their surface. Finally, the contraction of such muscle cells also requires the influx of sodium ions *via* specialised channels. With this signalling mechanism in mind, it becomes very easy to explain the devastating effects of the α-, μ-, and ω-conotoxins, which appear to function as follows.

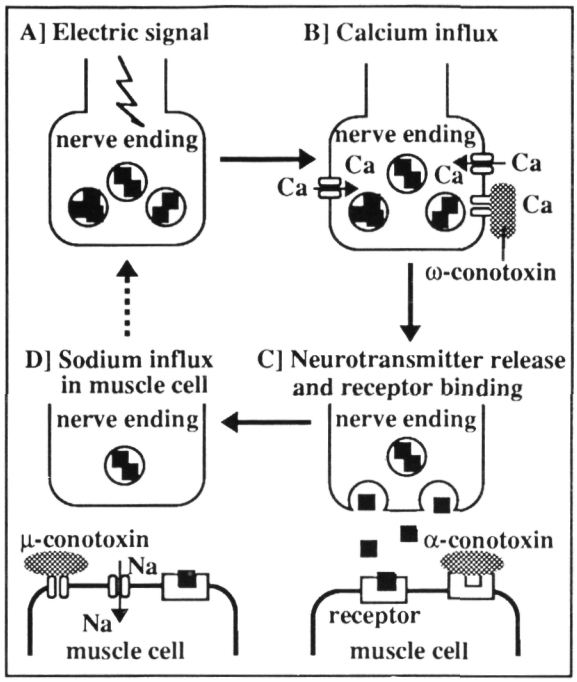

Fig. 2. Molecular mechanisms involved in the neuronal control of skeletal muscleaction: effect of conotoxins.

a) μ-Conotoxins block voltage-sensitive calcium channels at the nerve endings, thereby inhibiting the release of neurotransmitters.

b) α-Conotoxins block nicotinic acetylcholine receptors at the surface of skeletal muscle cells in a curare-like fashion, thereby causing paralysis and death in mice when the respiratorey muscles are affected.

c) ω-Conotoxins block sodium channels at the surface of the skeletal muscle cells, resulting in paralysis and death, as above.

Interestingly, these three classes of toxins appear to be small peptides, with only 13 to 29 amino acids (OLIVERA *et al.*, 1985). In nature, these peptides do not occur as loose amino acid chains. Instead, they are folded up in a well-defined fashion, and are stabilised by internal disulfide bonds (OLIVERA *et al.*, 1990). Although these three classes of toxins have been extensively investigated during the past few years, *Conus* venoms have been shown to possess many more toxin components. In this context, the venom of *C. geographus* has been demonstrated to contain dozens of different peptide components (OLIVERA *et al.*, 1990). When injected in mice, these peptides produce symptoms as varied as twisted jumping, head swinging, circular motion, sleep, paralysis, depressed activity, scratching and convulsions, and may even cause death. Some peptides produce even more exotic effects. For example, the "King Kong" peptide from *C. textile* makes lobsters assume a dominant posture. The molecular action mechanisms which are involved in the physiological effects of many of these peptide components are still unknown. Cone venoms thus contain a broad spectrum of biologically active peptides, and many of them even appear to be harmless. It is therefore not clear whether these peptide components are all really useful with respect to the feeding or the defense of the cone.

Conus toxins also interact with receptors on the cell membrane.

In addition to the peptide diversity within the venom of a single species, there is also a marked interspecies diversity in venom composition. These properties make *Conus* venoms a rich source of highly selective tools for investigating specific molecular mechanisms within a cell, and even for discriminating between such mechanisms (OLIVERA *et al.*, 1990). In addition, these venoms might also contain potentially useful drugs for medical treatments. So far, most of the attention has been devoted to the interaction between Conus toxins and cation conductance channels in the cell membrane (OLIVERA *et al.*, 1990). Since vasopressin-like peptides and nicotinic acetylcholine receptor antagonists were reported to be present in *Conus* venoms (OLIVERA *et al.*, 1985), we made the assumption that they might contain toxins which interact with other receptors as well.

To date, more than a hundred different molecules are known to possess a messenger function; they are classified either as neurotransmitters (released from nerve endings) or as

hormones (secreted from endocrine glands into the blood stream) (ALBERTS *et al.*, 1989). Each of these molecules is recognized by one or more specific receptors at the surface of responsive cells. Hence, our body also contains a great number of different receptors and, since they are ideal targets for medical drugs, it is of great interest to unravel their precise structure and molecular action mechanism. Previous research on receptors encompassed in isolated organs or *in vivo* experiments. However, it has now become possible to investigate them directly by binding of radioactively labelled hormone- or neurotransmitter analogs: i.e. the radioligands. Such in vitro radioligand binding experiments can be performed on cell membranes which are isolated from various tissues and organs. The desired tissues and organs are collected in a slaughter house (for calf material) or in a hospital (Akademisch Ziekenhuis- Vrije Universiteit Brussel, for post-mortem human material). With this novel approach, the inflicting of pain to animals as well as any unnecessary killing can thus be avoided.

During the past few years, we have devoted much of our attention to the identification and characterization of α_2-adrenergic receptors. They form part of a family of receptors (i.e. the adrenergic receptors) which recognize adrenaline. They exert a regulatory control of a wide range of physiological, behavioural and endocrinal functions, and are thought to play a role in conditions such as hypertension, anxiety and endogenous depression (RUFFOLO *et al.*, 1988). Because of our interest in α_2-adrenergic receptors, we first screened the venom of 22 *Conus* species for the presence of toxins which interact with such receptors (CZERWIEC *et al.*, 1989). The identification of such toxins was based on their ability to shield off the α_2-adrenergic receptors and, hence, to prevent the binding of a specific radioligand ([^{3}H]-idazoxan). The venoms of five *Conus* species (*C. planorbis* Born, 1778, *C. tessulatus*, *C. eburneus*, *C. textile* and *C. geographus*) were found to contain the desired toxin components. They produced a dose-dependent decrease in the binding of [^{3}H]-idazoxan to the α_2-adrenergic receptors in calf retina membranes. All other venoms tested were unable to affect binding of the radioligand and, hence, did not contain such toxins. Additional experiments revealed that the active toxins require the presence of calcium ions to shield off the α_2-adrenergic receptors, and that they are large peptides (with a molecular weight of more than 10.000 Dalton) (CZERWIEC *et al.*, 1989).

Conus toxins discriminate between related receptors.

Recent molecular cloning studies have shed light on hitherto unexpected structural relationships between hormone and neurotransmitter receptors. The α_2-adrenergic receptors are now recognised as making up part of a receptor superfamily, whose members are likely to constitute the descendants of a common ancestor (HARRISON *et al.*, 1991). The amino acid sequence of the α_2-adrenergic receptors is particularly close to that of one of the receptors which recognize the neurotransmitter serotonin: the 5-HT$_{1A}$ receptors. Despite this similarity in structure, α_2-adrenergic receptors and 5-HT$_{1A}$ receptors are each capable of selectively recognizing their own natural messenger molecules (i.e. adrenalin and serotonin, respectively). However, many synthetic analogs are unable to discriminate between both receptor types. A striking example is the radioligand [^{3}H]-rauwolscine, which was previously recognized to be selective for α_2-adrenergic receptors. However, we have shown that it binds with equally high affinity to the 5-HT$_{1A}$ receptors in the human frontal cortex (CONVENTS *et al.*, 1989). In this tissue, [^{3}H]-rauwolscine can label both receptor types together.

The experiment presented below reveals that conotoxins may help us in discriminating between α_2-adrenergic receptors and 5-HT$_{1A}$ receptors. The experiment comprises the incubation of calf frontal cortex membranes with a

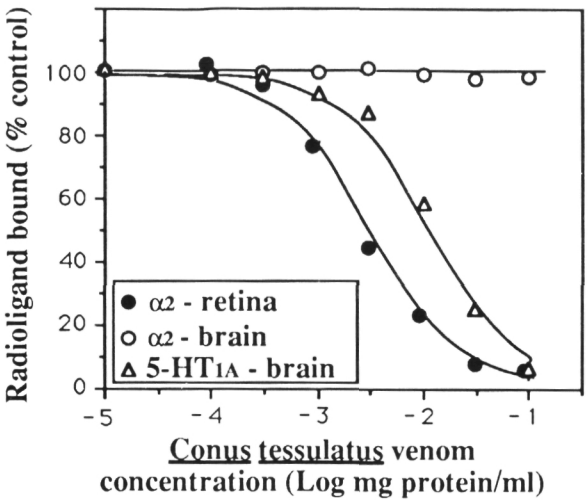

Fig. 3. Effect of increasing concentrations of *Conus tessulatus* venom on [^{3}H]rauwolscine binding to α_2-adrenergic and 5-HT_{1A} serotoninergic receptors in calf retina and brain. Values are means of two (for brain) or three (for retina) determinations with S.D.<10%.

fixed concentration of [^{3}H]-rauwolscine, either in the presence of serotonin (which blocks the 5-HT_{1A} receptors, so that the radioligand only labels α_2-adrenergic receptors) or of adrenalin (which blocks the α_2-adrenergic receptors, so that the radioligand only labels 5-HT_{1A} receptors). The addition of increasing concentrations of the venom of Conus tessulatus (shown as the abscissa, logarithmic scale, Fig. 3) to this mixture provokes a net concentration-dependent decrease in [^{3}H]-rauwolscine binding to the 5-HT_{1A} receptors (Fig. 3, open triangles). Binding to the α_2-adrenergic receptors in brain is unaffected by the venom (Fig. 3, open circles). This contrasts with the venom's capability to decrease [^{3}H]-rauwolscine binding to the α_2-adrenergic receptors in calf retina (Fig. 3, solid circles).

Taken together, these data reveal that the venom of *C. tessulatus* contains one or more toxins which interact with 5-HT_{1A} receptors but not with α_2-adrenergic receptors in calf cortex membranes. A similar discrimination between both receptor types by the venom of *C. tessulatus* has recently also been observed in human frontal cortex membranes (DE VOS *et al.*, 1991). Whereas the natural messenger molecule serotonin and the vast majority of synthetic analogs produce only transient shielding of the 5-HT_{1A} receptors, the toxins present in the venom of *C. tessulatus* appear to do so in a permanent way.

Experiments such as that with results as shown in Fig. 3 give rise to two following conclusions. Firstly, it is evident that *Conus* venoms contain toxins which may be used as tools for discriminating between certain structurally related receptors. (In addition to the data above, we have also observed that the venoms of *C. tessulatus* and *C. textile* contain components which discriminate between D1 and D2 dopamine receptors and between M1 and M2 muscarinic receptors, data to be published). Secondly, experiments with *Conus* toxins (and with plant and animal toxins in general) may raise new questions to be asked about certain cellular processes, and may lead to a better insight into their complex nature. The lack of effect of *C. tessulatus* venom on the α_2-adrenergic receptors in calf cortex membranes is indeed in marked contrast with its ability to affect α_2-adrenergic receptors in retina membranes. A possible explanation for this discrepancy is that the α_2-adrenergic receptors in calf retina are structurally different from those in brain. The existence of different α_2-adrenergic receptor subtypes has recently been reported (BYLUND *et al.*, 1988) and, at present, we are trying to find out whether there is a relationship between this receptor heterogeneity and the discrepant behaviour of the *C. tessulatus* venom. Future directions in our research on *Conus* toxins involves the purification of toxins which interact with neurotransmitter receptors, and the unraveling of their structure. These studies may give rise to the production of genetically engineered conotoxins in the future.

Materials and Methods

[^{3}H]Rauwolscine (74 Ci/mmol) was obtained from New England Nuclear (Boston, MA). Calf retina and brain were obtained from a local sloughter house, and membranes were prepared as described (CZERWIEC *et al.*, 1989; DE VOS *et al.*, 1991). Binding experiments with [^{3}H]rauwolscine were performed as described (DE VOS *et al.*, 1991). Frozen specimens of Conus tessulatus were obtained from the Seychelles and stored at -20°C until use. The venom was obtained by homogenizing the dissected ducts, follwed by sonication, and partially purified by centrifugation (CZERWIEC *et al.*, 1989).

Acknowledgements

G.V. is Onderzoeksdirecteur of the Nationaal Fonds voor Wetenschappelijk Onderzoek, Belgium. This work was supported by the Fonds voor Geneeskundig Wetenschappelijk Onderzoek, Belgium. We are very grateful to Mr. G. Van Geel, V.V.O.B. The Seychelles, for sending us specimens of Conus tessulatus, and to Mrs. I. Van Liefde for translatory work.

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